Evaluation of an Enzyme-Linked Immunosorbent Assay, Direct Immunofluorescent Filter Technique, and Multiplex Polymerase Chain Reaction for Detection of *Escherichia coli* O157:H7 Seeded in Beef Carcass Wash Water†

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ABSTRACT

In commercial beef processing, carcasses are customarily washed with water to remove physical and microbial contamination. Assaying the water that is shed from the carcasses after washing is a convenient method to determine whether the carcass is contaminated with *Escherichia coli* O157:H7 or other bacterial pathogens. *E. coli* O157:H7 was inoculated into carcass wash water at various levels and the bacteria were then concentrated by filtration. After collection of bacteria in the filter units, the nylon membranes were cut out and placed in tubes containing growth medium, and the tubes were mixed vigorously to dislodge the bacteria from the membranes. Prior to enrichment, samples were removed for testing by a multiplex polymerase chain reaction (PCR) and a direct immunofluorescent filter technique (DIFT). The remaining samples were subjected to 4-h enrichment culturing at 37°C, after which aliquots were removed for testing by multiplex PCR, DIFT, and an enzyme-linked immunosorbent assay (ELISA). Following 4-h enrichment culturing, *E. coli* O157:H7 was detected in wash water samples initially inoculated with ca. 100, 0.1, and 1 CFU/ml by ELISA, DIFT, and multiplex PCR, respectively. Testing of the wash water using the ELISA and the DIFT can be accomplished in less than 8 h. On the basis of these results, assaying carcass wash water by ELISA, DIFT, or multiplex PCR can be useful for detection of *E. coli* O157:H7 beef carcass contamination and can potentially be employed to identify carcasses for further processing to inactivate the organism.

During the beef slaughtering process, *Escherichia coli* O157:H7 can be accidentally introduced onto carcass surfaces, in particular during hide removal and evisceration. Beef carcasses are commonly trimmed and spray washed to remove physical and microbial contaminants. Spray washing is effective in reducing bacterial numbers on meat surfaces *(5)*. Gorman et al. *(5)* achieved approximately 2–log CFU/cm^2 reductions in total plate bacteria counts on spray-washed beef tissue inoculated with a bovine fecal paste containing *E. coli*. It would be expected that bacteria removed from meat tissue by spray washing could be recovered in the water that is shed from carcasses after they are washed. Several methods have been described for testing carcasses for the presence and levels of bacteria, including direct agar contact, excision, and swabbing using cheesecloth, sponges, or wooden swabs *(3)*. With many of these methods, however, areas of bacterial contamination may be missed since the entire carcass surface is not sampled. Testing wash water shed from carcasses allows sampling of the entire surface for the presence of target pathogenic bacteria. If the bacteria are present in the wash water, it is likely that a portion will remain on the carcass. Thus, contaminated carcasses can be identified and possibly earmarked for processing that includes heat inactivation steps. *Campylobacter* and *Salmonella* were detected by various methods in chicken carcass rinses *(6, 14)*; however, there have been no reports on detection of microbial pathogens in beef carcass rinse water.

In recent years, a number of immunologic and nucleic acid–based systems have been developed for detection and/or identification of *E. coli* O157:H7 in fecal specimens and in food samples *(1, 2, 12)*. Immunologic methods involve binding of an antibody to an antigen; the enzyme-linked immunosorbent assay (ELISA) is the most commonly used immunologic technique for rapid detection of pathogenic bacteria and toxins. Nucleic acid–based systems, in particular polymerase chain reaction (PCR) amplification techniques, are relatively rapid, show a high degree of specificity, and are extremely sensitive. The objective of this study was to develop and evaluate a system to recover *E. coli* O157:H7 from beef carcass wash water and to screen for the organism using an ELISA, a direct immunofluorescent filter technique (DIFT), and a multiplex PCR assay.

MATERIALS AND METHODS

Bacteria and beef carcass wash water. *E. coli* O157:H7 B1409 (produces Stx2) was obtained from the Centers for Disease

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Control and Prevention (Atlanta, Ga.). The culture was maintained on tryptic soy agar (Difco Laboratories, Detroit, Mich.) at 4°C. A loopful of culture was inoculated into 50 ml of nutrient broth (Difco) and was grown at 37°C for 18 h with aeration. Tenfold serial dilutions of the culture were prepared in 0.1% peptone (Difco) and were used for the carcass wash water inoculations. To obtain the number of CFU per milliliter, dilutions were plated onto nutrient agar (Difco) using a spiral plater (Model D, Spiral Systems, Inc., Bethesda, Md.), and colonies were counted using a laser counter (Spiral Systems, Inc.). Carcass wash water was obtained from a local beef slaughter plant. After carcasses were split and washed (Chad washing system, 250 psi spray pressure [17.2 bars]) with potable water at room temperature, the water that was shed from the split carcasses was collected into sterile plastic containers with the aid of a large funnel. The wash water was refrigerated for approximately 24 h and was then divided into smaller portions and frozen at −20°C prior to use.

Carcass wash water inoculation and processing. The procedure employed for testing the wash water is depicted in Figure 1. The wash water was first filtered through light-duty, lint-free paper wipers (11 lb, Macalaster Bicknell, Millville, N.J.) placed in a funnel to remove large pieces of fat and other material. The water was then divided into 300-ml portions, and *E. coli* O157:H7 was added at various concentrations ranging from 0.1 to 1,000 CFU/ml of wash water. The negative control samples were not inoculated with *E. coli* O157:H7. The water was then filtered through 2-μm glass fiber prefilters (Fisher Scientific, Pittsburgh, Pa.) placed in porcelain Büchner filter funnels. Finally, the water was filtered through 0.2-μm Nalgene nylon filters (A. Daigger & Company, Inc., Wheeling, Ill.). The membranes were cut from the filters using a sterile dissecting scalpel and, using sterile tweezers, were placed into 15-ml volume tubes with 5 ml of modified EC broth containing novobiocin (0.02 mg/ml) *(8)*. The tubes were mixed intermittently for 2 min to dislodge bacteria from the membranes. Prior to enrichment, samples were removed for testing by DIFT and PCR. The remaining sample was subjected to enrichment culturing for 4 h at 37°C at 200 rpm, after which samples were removed for testing by ELISA, DIFT, and multiplex PCR assay.

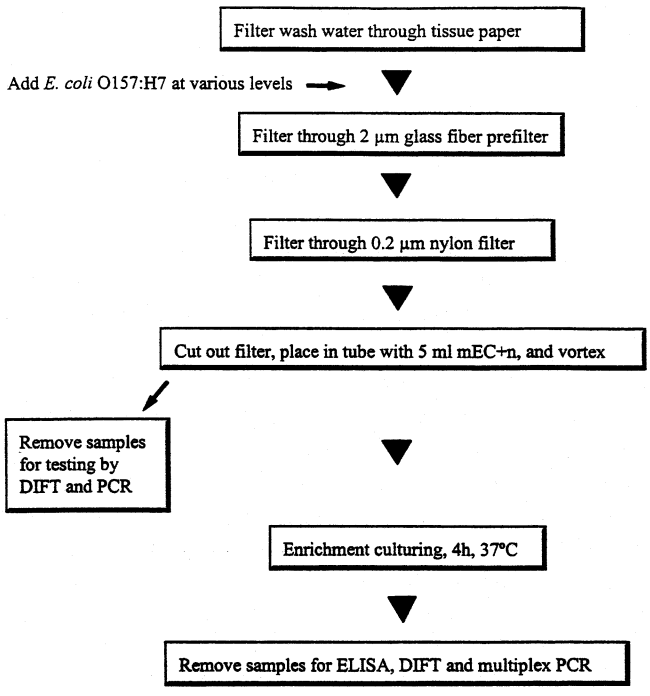

FIGURE 1. *Flow chart showing procedure used to process and test beef carcass wash water.*

ELISA. The EHEC-Tek Test System for *E. coli* O157:H7 (Organon Teknika Corporation, Durham, N.C.) was used for detection of the organism in artificially inoculated wash water; however, several modifications of the procedure recommended by the manufacturer were made. Briefly, 100 μl of nonheated enrichment samples was added to the wells, and the plate was incubated for 30 min at 37°C. After washing three times with wash solution, 100 μl of conjugate solution (both solutions provided with kit) was added, and incubation was continued for 30 min at 37°C. The wells were washed six times with wash solution, and 100 μl of 3,3′,5,5′ tetramethylbenzidine peroxidase enzyme substrate was added. Following 30 min of incubation at ambient temperature, 100 μl of stop solution (provided with kit) was added to each well, and the plate was read immediately using an EL 312 microplate reader (BIO-TEK Instruments, Winooski, Vt.) set at an absorbance of 450 nm. The determination of the positive cutoff value was performed as recommended by the manufacturer. Briefly, a value of 0.250 is added to the average of the absorbance readings of the two negative control samples, which are included in each assay. Positive cutoff values obtained were in the range of 0.351 to 0.377. A presumptive positive sample, thus, would have an absorbance reading greater than or equal to the cutoff value. The absorbance value of the positive control samples should be greater than 2.000.

DIFT. Samples prior to enrichment and enrichment cultures (500 μl) were filtered through black polycarbonate membranes, 25 mm in diameter and with a pore size of 0.2 μm (Poretics Corporation, Livermore, Calif.), placed in fritted-glass funnels. The vacuum was removed, and bacteria captured on the membrane were reacted with 150 μl of fluorescein isothiocyanate–labeled antibody reactive against *E. coli* O157 (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) diluted to a concentration of 50 μg/ml in phosphate buffered saline (PBS), pH 7.2. Following incubation for 30 min in the dark at ambient temperature, the membranes were washed twice with 500 μl of PBS and then were placed on glass slides, and a drop of 60% glycerol was added prior to covering with a glass coverslip. The membranes were examined by fluorescence microscopy using an Olympus BH-2 microscope (Olympus Corporation of America, New Hyde Park, N.Y.) and photographed using Kodak TMAX 100 film.

PCR. Five microliters of the samples prior to enrichment and enrichment samples were subjected to multiplex PCR amplification as described previously *(2)* with the use of GeneReleaser. The primer sets for amplification of plasmid and SLT genes (currently called *stx*) were the same as those described previously *(2)*. For amplification of the *eaeA* gene sequence, primer AE20 (reverse primer, 3′ end of sequence) was also the same as that described previously, except that the first T at the 5′ end was replaced with an A (new primer designated AE20-2). A different forward primer was used to obtain an amplification product smaller than 1,089 bp. The sequence of this primer, AE22, was 5′-ATTACCATCCACA-CAGACGGT-3′. The PCR product obtained with primer set AE22 and AE20-2 for amplification of the *eaeA* gene sequence was 397 bp in size. Products of the multiplex PCR were analyzed by agarose gel electrophoresis (1.5%) followed by ethidium bromide staining of the DNA.

RESULTS AND DISCUSSION

Three types of methods, ELISA, DIFT, and multiplex PCR assay, were evaluated in terms of sensitivity, rapidity,

and ease of performance for detection of *E. coli* O157:H7 in beef carcass wash water. To detect low levels of *E. coli* O157:H7, the wash water samples were concentrated by filtration. This procedure allowed recovery of up to 60% of the inoculated bacteria. Also, during the enrichment step, while the sample tubes were rotated at 200 rpm, additional bacteria were probably dislodged from the membranes. The wash water was initially filtered through lint-free paper to remove particulate matter. However, there was little particulate matter present, and this initial filtering step could possibly be eliminated. The inoculum was added after this filtering step since it was determined that the number of *E. coli* O157:H7 in the wash water prior to and after filtering was essentially identical. The number of *E. coli* O157:H7 in the filter-concentrated samples increased by approximately 2 to 2.5 log CFU/ml following 4-h enrichment culturing in modified EC broth containing novobiocin. By plating on both brain heart infusion agar and MacConkey sorbitol agar, the level of background organisms in the wash water was determined to be approximately 1×10^1 CFU/ml.

ELISA. The EHEC-Tek microelisa wells are coated with polyclonal antibodies to the lipopolysaccharide antigen of *E. coli* O157:H7, and the second antibody is a horseradish peroxidase–labeled monoclonal antibody (4E8C12) that reacts with two outer membrane proteins expressed by *E. coli* O157:H7 and O26:H11 *(9)*. Since, as with many immunoassays, nonspecific reactions can occur due to cross-reactivity of the antibody with antigenically similar organisms, samples that generate absorbance values above the cutoff value are considered presumptive positives. Confirmatory testing of the presumptive positive samples is required. A report on the use of the EHEC-Tek immunoassay for detection of *E. coli* O157:H7 in ground beef inoculated at levels of 50, 5, and 0.5 CFU/g showed favorable agreement between the immunoassay and immunomagnetic separation followed by plating onto cefixime-tellurite-sorbitol MacConkey agar *(1)*. In the present study, carcass wash water had a very low level of background microflora (ca. 1×10^1 CFU/ml) and did not contain materials that are found in foods, such as ground beef, that could possibly be inhibitory to immunoassays. Therefore, immunocapture using magnetic beads followed by overnight enrichment was not performed as recommended by the manufacturer of the EHEC-Tek immunoassay. Assay time was, thus, notably shortened. The detection limit using the ELISA was $\leq$100 CFU/ml of wash water (Fig. 2). The samples were subjected to only 4 h of enrichment culturing to allow performance of the entire assay in less than 8 h. Obviously, increasing the enrichment period from 4 to 18 or 24 h would considerably improve the limit of detection of the ELISA and also of the DIFT and multiplex PCR assay.

DIFT. The concentration of bacteria by filtration onto a membrane, followed by the use of fluorescent stains for direct visual detection of the organisms by epifluorescent microscopy, is a technique that has been applied to foods, especially to milk testing. When applied to milk, following filtration, the membrane is stained with acridine orange and viable bacteria appear orange colored *(10)*. This procedure,

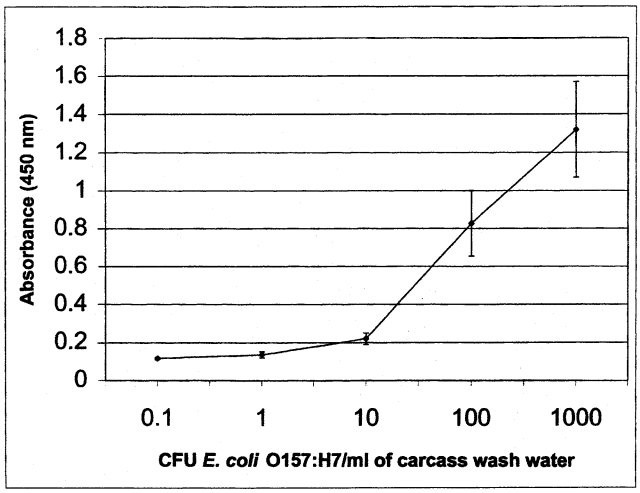

FIGURE 2. *Sensitivity of modified EHEC-Tek ELISA for detection of* E. coli *O157:H7 in beef carcass wash water following 4-h enrichment culturing in modified EC broth containing novobiocin. Results shown are of four separate experiments. The positive cutoff value at 450 nm was in the range of 0.351 to 0.377.*

which is usually employed for enumeration but not for identification of bacteria, is called the direct epifluorescent filter technique. The DIFT (also called antibody–direct epifluorescent filter technique) employs fluorescent antibody conjugates reactive against target organisms and, thus, can be used to detect and enumerate specific pathogens *(13)*.

In the present study, the limit of detection using the DIFT on samples that had undergone 4 h of enrichment culturing was $\leq$0.1 CFU/ml (six to twelve bacteria per microscopic field using a 40$\times$ objective lens) of wash water and was $\leq$10 CFU/ml (one fluorescent *E. coli* O157:H7 visible in ca. 50% of microscopic fields examined using a 40$\times$ objective lens) using samples that had not undergone enrichment. Fluorescent bacteria captured on the membranes were clearly visible microscopically (Fig. 3). The

FIGURE 3. *Photomicrograph of* E. coli *O157:H7 reacted with anti-O157 conjugated to fluorescein isothiocyanate and analyzed by DIFT. The wash water sample was inoculated with approximately 0.3 CFU of* E. coli *O157:H7 per ml, and the sample was subjected to 4-h enrichment culturing in modified EC broth containing novobiocin. The membrane was examined using a 40$\times$ objective lens.*

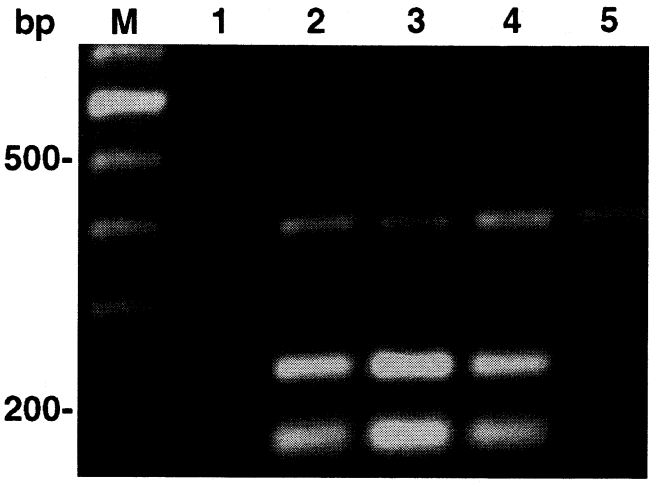

FIGURE 4. *Agarose gel stained with ethidium bromide showing PCR products from amplification of the plasmid (166 bp),* stx_2 *(225 bp), and* eaeA *(397 bp) DNA sequences. The wash water samples were subjected to 4-h enrichment culturing in modified EC broth containing novobiocin. Lane M, DNA size markers (1-kb ladder; GIBCO BRL, Gaithersburg, Md.); lane 1, uninoculated negative control; lanes 2 through 5, samples were inoculated with ca. 1,000, 100, 10, and 1 CFU of* E. coli *O157:H7 per ml of wash water, respectively.*

DIFT was simple, rapid, and sensitive and the entire assay, including enrichment culturing, can be performed in less than 6 h. However, since the fluorescein isothiocyanate–labeled antibody that was used reacts with the O157 lipopolysaccharide antigen, only presumptive positive results are obtained. Confirmatory testing of the sample would be required to determine the H flagellar antigen type.

Multiplex PCR. We have previously reported on the use of a multiplex PCR for direct detection of *E. coli* O157:H7 in enrichment cultures of ground beef and raw milk *(4)* and for identification of suspect colonies on selective/differential medium *(2)*. The use of a different forward primer for amplification of the *eaeA* gene sequence resulted in a smaller PCR product than that previously described *(2, 4)*. In a multiplex PCR, more efficient amplification is achieved if the sizes of the amplified DNA products are close to one another. The limit of detection using the multiplex PCR assay on samples that had undergone 4 h of enrichment culturing was 1 CFU/ml (Fig. 4, lane 5). Products resulting from amplification of plasmid sequence (166 bp), stx_2 (225 bp), and *eaeA* (397 bp) genes were visible. *E. coli* O157:H7 was detectable in samples prior to enrichment at a level of 100 CFU/ml of wash water (data not shown). Since the multiplex PCR is specific for *E. coli* O157:H7 and Stx-producing *E. coli* O157:NM or H^- *(2)*, further confirmatory testing could be kept to a minimum. The multiplex PCR can be used as a confirmatory assay on presumptive positive ELISA or DIFT samples. The samples can also be plated onto selective media and suspect colonies identified using the multiplex PCR.

It would be possible to filter concentrate a larger volume of wash water (e.g., 1 liter), thus collecting a higher number of target bacteria and potentially improving the sensitivity of the ELISA, DIFT, and multiplex PCR assay. *Salmonella* was recovered from 1-liter volumes of filter-concentrated environmental water samples and detected using a multiplex PCR approach *(15)*. Hoszowski et al. *(6)* used a series of filtration steps, 18-h selective enrichment, and colony blot immunoassay to identify and enumerate captured *Salmonella* in 400-ml volume chicken carcass rinse samples. The limit of detection of this method was 5 CFU/carcass rinse sample. In the present study, the DIFT was the most sensitive method; however, it should only be used as a screening assay since presumptive positive results are obtained. If antibodies that are *E. coli* O157:H7–specific become available and are used in DIFT assays, the number of false-positive results obtained would be reduced, as would the amount of confirmatory testing. Microscopic examination of numerous samples can be very tedious; however, developments in automated systems for image analysis should further simplify the performance of DIFT assays and improve sensitivity *(7, 11)*. The multiplex PCR is sensitive and the most specific for *E. coli* O157:H7 of the three methods tested since specific virulence genes are targeted for amplification. The ELISA, DIFT, and multiplex PCR are simple and rapid and should potentially be amenable for testing of carcass wash water for the presence of *E. coli* O157:H7.

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